miRNA-facilitated self replicative RNA (srRNA) reprogramming of endothelial progenitor cells (EPCs) derived from human peripheral blood and cord blood

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Introduction

Peripheral blood provides easy access to adult human cell types for reprogramming purposes. In late 2012, two groups demonstrated the effective isolation, expansion, and subsequent generation of retrovirally-induced iPS cell lines from endothelial progenitor cells (EPCs) derived from human peripheral blood[1,2]. While circulating EPCs are a rare population of cells, we effectively isolated and expanded multiple adherent EPC lines. These primary EPC cultures can be established from fresh and frozen human peripheral blood as well as cord blood samples, some from as little as 1 x 10^7 mononuclear cells (MNCs) (Figure 1). The EPCs adherent nature and high proliferative capacity while maintaining their cell identity makes them highly desirable for transfection, and ultimately reprogramming into iPS cells with RNA.

In 2013, published results demonstrated the reprogramming of human neonatal fibroblasts into iPS cells using a polycistronic self-replicative RNA (srRNA)[3], with as few as one transfection. Subsequently, we have extended and improved the application of srRNA for cellular reprogramming to peripheral blood-derived EPCs, cord blood derived EPCs, as well as adult fibroblasts. The generation of srRNA-EPC-iPS cells required optimization of mRNA delivery, culture media composition and transitions, as well as incorporation of reprogramming associated microRNAs. These improvements resulted in a simple two transfection, no-split protocol on extracellular matrix without the need for conditioned medium (Figure 2) for the establishment of integration-free, wholly pluripotent human iPS cell lines (Figure 3) from multiple target cell types (Table1). Additionally, iPS cell lines derived from srRNA reprogramming of EPCs exhibit unique innate genetic stability (Tables 2 and 3) when compared to previously published results of lines derived from fibroblasts or lines derived using integrating reprogramming technologies[4,5], making them an exceptional choice for cell fate manipulations and applications requiring clinical grade cells. Lastly, generation of these clinically relevant EPC-iPS cells using this novel srRNA reprogramming technology presents a therapeutic opportunity to specifically treat myeloproliferative disorders in which the disease-causing somatic mutations are restricted to cells in the hematopoietic lineage.

Establishment and immunocytochemical characterization of EPCs derived from peripheral blood

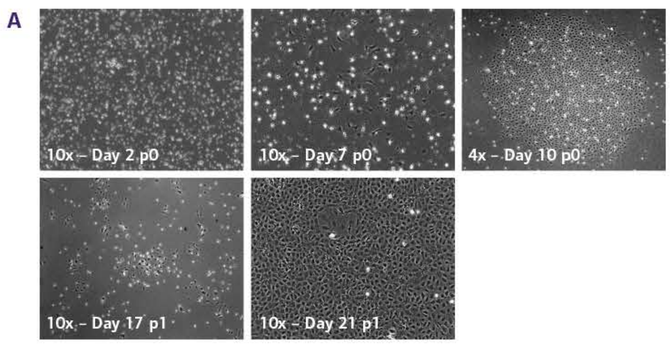


FIGURE 1A: Derivation timeline of adherent EPCs from peripheral blood.
A minimum of 1x10^7 human mononuclear cells (MNCs) were seeded into a single T75 flask coated with collagen I and cultured at 37°C, 5% CO_2, and 21% O_2 in Lonza EGM-2 medium, where supplied FBS was replaced with HyClone™ ES-qualified, defined FBS to 20% v/v final.

Day 2 → Primary (p0) suspension culture of human mononuclear cells.
Day 7 → Emerging EPC colonies.
Day 10 → Maturation of EPC colony.
Day 17 → p1 EPC culture 3 days after passaging.
Day 21 → Confluent p1 EPC culture ready to be passaged.

FIGURE 1B: Immunocytochemistry (ICC) of EPCs. EPCs at p5 were seeded at a density of 5 x 10^4 cells per well of a 12-well plate. Cells were fixed the following day with 4% paraformaldehyde (PFA) and stained with the appropriate antibodies and DAPI for visualization. Merged images are shown.

Protocol timeline and morphology progression for reprogramming EPCs using Stemgent microRNA and self-replicative RNA (OKSiM)

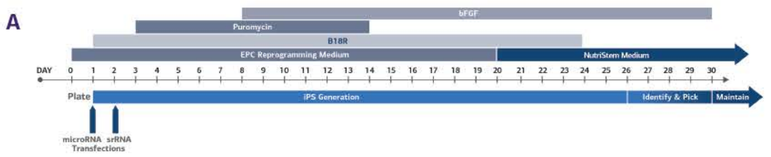


FIGURE 2A: Timeline for the reprogramming of human EPCs using Stemgent StemRNA-SR Reprogramming Kit (Cat. No. 00-0075) with non-modified srRNA and microRNA. RNA transfections and puromycin selection were carried out in Lonza EGM-2 medium. Culture medium was transitioned to NutriStem® XF/FF Culture Medium on Day 20 prior to B18R removal on Day 24.

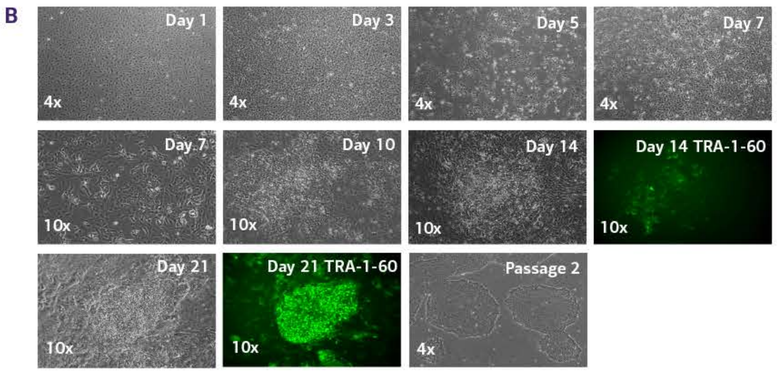
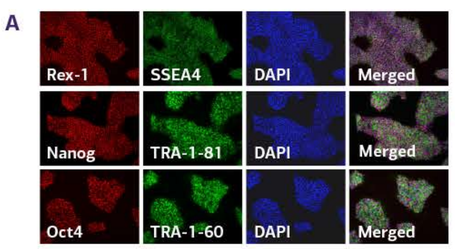


FIGURE 2B: Primary reprogramming culture morphology progression, resulting from the reprogramming of peripheral blood derived EPCs (Donor 6-1) with Stemgent StemRNA-SR Reprogramming Kit (Cat. No. 00-0075). Day 0: EPCs (p6) were seeded at 2 x 10^5 cells per well of a 6-well Corning Matrigel®-coated plate. Day 1: EPCs were transfected with 70 pmol of microRNA. Day 2: EPCs were transfected with 1 µg of srRNA (OKSiM). Days 0-19: culture was maintained at 37°C, 5% CO_2, and 5% O_2 in Lonza EGM-2 medium with puromycin (0.4 µg/ml) selection from Days 3-14. Day 20: culture medium was changed to NutriStem XF/FF Culture Medium. Recombinant B18 protein was included in culture medium from Days 0-24. iPS cell morphologies emerge as early as Day 14 and are able to be isolated by Day 26. Day 14 and Day 21 primary iPS cell colonies were identified using Stemgent StainAlive™ TRA-1-60 antibody.

Characterization of EPC srRNA iPS cell line (Donor 6-1) derived using Stemgent StemRNA-SR Reprogramming Kit



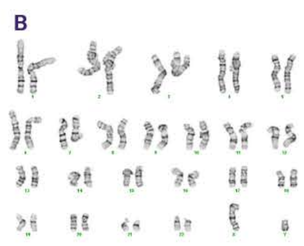


FIGURE 3A: Pluripotency immunocytochemistry (ICC) of EPC-srRNA-iPS cell line (Donor 6-1: p13) derived from human EPCs (p5) and expanded on Corning Matrigel with NutriStem XF/FF Culture Medium. Nuclear pluripotency staining with Rex1, Nanog, and Oct4 antibodies is seen in red. Cell surface pluripotency staining with SSEA4, TRA-1-81, and TRA-1-60 antibodies is seen in green. Merged images are shown.

FIGURE 3B: Normal karyotype of EPC-srRNA-iPS cell line (p9) expanded on Corning Matrigel with NutriStem XF/FF Culture Medium.

FIGURE 3C: Histological analysis of teratoma resulting from the injection of EPC-srRNA-iPS cells (Donor 6-1: p13) into the kidney capsule of NOD-Scid mice. Prior to injection, EPC-srRNA-iPS cells (Donor 6-1) expanded on Corning Matrigel with NutriStem XF/FF Culture Medium.

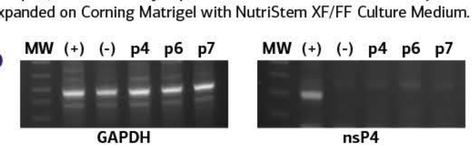


FIGURE 3D: RT-PCR analysis of EPC-srRNA-iPS cell line for cytoplasmic retention of polycistronic srRNA. EPC-srRNA-iPS cell line (Donor 6-1) was expanded on Corning Matrigel with NutriStem XF/FF Culture Medium. Total RNA was isolated from: p4, p6, and p7 of the iPS cell line; non-transfected EPCs [(-) control] and EPCs transfected with 1 µg of polycistronic srRNA [(+) control]. Load control primers = GAPDH. Polycistronic srRNA specific primers = nsP4 (non-structural protein 4).

BLOOD SOURCE	PRIMARY EPC ESTABLISHMENT EFFICIENCY*	REPROGRAMMING EFFICIENCY (PATIENT TO PATIENT)	REPROGRAMMING EFFICIENCY (PER WELL)
Peripheral Blood	18/22 = 82%	9/13 = 70%	0.0015-0.0025%
Cord Blood	46/53 = 87%	33/41 = 81%	0.005-0.01%
TOTAL	64/75 = 85%	42/54 = 78%	-

TABLE 1. Primary EPC establishment and subsequent reprogramming efficiencies for srRNA reprogramming of EPCs from human blood samples. Primary EPC establishment efficiency for peripheral blood is cumulative of both fresh blood and cryopreserved MNC samples. Cord blood efficiency data are from cryopreserved MNCs only. All samples had a minimum of 5x10^7 MNCs for seeding onto collagen coated T-75 flask. Reprogramming efficiencies (patient to patient or per well) generated using presented two transfection protocol.

iPS CELL LINE	SAMPLE	AUTOSOMAL CNVS IN iPSC	CHROMOSOME	SIZE OF REGION COVERED BY CNVS	COPY GAIN OR LOSS	GENES INVOLVED
EPC-srRNA	patient 1	1	16	12806	single copy loss	TRAF7
	patient 2	0				
	patient 3	0				
	patient 4 (D)	0				
	patient 5	0				
	patient 6-1 (D)	0				
	patient 6-2 (D)	1	6	50160	single copy loss	KHDRBS2 | LOC100128610
	patient 6-3 (D)	0				
EPC-retro	patient 6-1 (D)	3	6	105930	single copy loss	KHDRBS2 | LOC100128610
			6	233158	single copy loss	KHDRBS2 | LOC100128610
			13	47059	single copy loss	LOC730236 | KLHL1
	patient 6-2 (D)	3	3	346679	double copy loss	LOC255025 | LOC644063
			5	64569	double copy loss	LOC100287592 | LOC642366|LOC642366|ISL1|ISL1 | LOC100287776
			6	52655	single copy loss	GRIK2 | LOC100132919
	patient 5	2	3	82105	double copy loss	ZIC4|ZIC4 | ZIC1|ZIC1|ZIC1 | FLJ30375|FLJ30375 | AGTR1
			3	148326	single copy loss	C3orf38 | EPHA3
Fibroblast-srRNA	patient 1 -1	3	5	190516	double copy loss	CDH9
			20	159147	double copy loss	MACROD2
			7	145891	double copy loss	SEMA3A
	patient 1 -2	1	2	3490	double copy loss	CDH9

TABLE 2. Copy number variation (CNV) comparison of EPC and fibroblast iPS cell lines generated using srRNA or retroviral-mediated reprogramming factor delivery. CNV data generated on the Illumina Human CytoSNP-12 DNA Analysis BeadChip platform. For CNV calls, 5kb size cut-off value and a minimum of ten markers (SNPs) were used as analysis configurations.

	NUMBER OF iPS CELL LINES ANALYZED	TOTAL CNVS	AVERAGE CNVS
EPC-srRNA-iPSCs	8	2	0.25
EPC-retro-iPSCs	3	8	2.67
srRNA-Fibroblast-iPSCs	2	4	2

TABLE 3. Summary of CNV data generated on the Illumina Human CytoSNP-12 DNA Analysis BeadChip platform.

Summary

- EPCs are a more genetically stable target cell type than fibroblasts for cellular reprogramming
- srRNA-EPC-iPS cell lines exhibit greater genetic stability than retrovirus-EPC-iPS cell lines
- Simplified reprogramming protocol using **Stemgent StemRNA-SR Reprogramming Kit (Cat. No. 00-0075)**
  - 200,000 primary EPCs seeded onto Corning Matrigel
  - Requires only 2 transfections (microRNA + srRNA)
  - No B18R mRNA cotransfection or conditioned medium required – uses B18R protein
  - No reprogramming culture passaging/manipulation
  - 3+ weeks for iPS cell colony establishment
  - Polycistronic srRNA cleared from isolated iPS cell lines in 3-4 passages (2 weeks)
- **Simple, efficient primary EPC line establishment (85%)**
  - Human peripheral and cord blood samples
  - Fresh or frozen samples
  - Two week primary culture establishment.

References

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